EPR spin-trapping studies of radicals generated from the Fe^{II}-catalysed degradation of nucleobase, nucleoside, RNA and DNA hydroperoxides †

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Nucleobase, nucleoside, RNA and DNA hydroperoxides have been generated by exposure of the parent compounds to high energy electrons in the presence of oxygen; EPR spin-trapping experiments using 2-methyl-2-nitrosopropane (MNP) and 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO) have been employed to study the reactions of alkoxyl radicals generated from their reaction with Fe^{2+}. Alkoxyl radicals generated from the pyrimidine hydroperoxides (nucleobases and nucleosides) are shown to be capable of reacting with a variety of substrates, which include the pyrimidine nucleobases and nucleosides themselves and histone proteins. Attack on the parent pyrimidine compounds involves addition to the C(5) and C(6) atoms of the pyrimidine ring; reaction with the histone proteins, amino acids and peptides gives carbon-centred species, providing direct evidence for transfer of damage *via* hydrogen-atom abstraction. Rapid reactions with antioxidants are also demonstrated.

Introduction

It is widely recognised that the action of ionising radiation on biological systems can generate free radicals *in vivo*, and hence cause DNA damage.[1–4] It is also well established that when DNA nucleobases and nucleosides are irradiated in the presence of oxygen, hydroperoxides can also be formed;[3,5–10] protein, lipid and small alkyl hydroperoxides can also be formed in an analogous manner, and there is considerable evidence to suggest that damaging radicals may be formed from their decomposition.[11–13]

Of the bases studied, thymine has been suggested to be most susceptible to the effects of ionising radiation,[3,6–10] generating mixtures of hydroperoxides in which 6-hydroperoxy-5-hydroxy-5,6-dihydrothymine (6-TOOH) and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine (5-TOOH) have been identified as the major products.[7–10] These hydroperoxides have both been shown to react in the presence of transition metals such as Fe^{2+} and Cu^{2+} to form thymine glycol;[8–10] the hydroperoxides are also mutagenic,[14,15] an effect which is enhanced by the presence of metal ions including Fe^{2+} and Cu^{2+}. These observations are consistent with the generation of free radicals from the hydroperoxides.[1]

Relatively little is known about the mechanisms of these metal-ion induced degradation reactions, which free radicals, if any, are involved in these reactions and the potential of these radicals to damage DNA and other cellular constituents. In the investigation to be described here we set out to generate hydroperoxides by irradiation of the pyrimidine and purine nucleobases and nucleosides, as well as RNA and DNA, under aerobic conditions. It was then intended to investigate the reaction of the hydroperoxides with Fe^{2+}, by use of EPR spin-trapping techniques using the spin traps 2-methyl-2-nitrosopropane (MNP) and 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO), and thereby examine the nature of any hydroperoxide-derived radicals and their reactions with DNA, histone proteins, selected amino acids, peptides and antioxidants.

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Results and discussions

(a) Generation of pyrimidine (nucleobase and nucleoside) hydroperoxides

Hydroperoxides were generated by exposure of stock solutions of pyrimidine nucleobases and nucleosides (typically 1×10^{-2} mol dm^{-3}) to high energy electrons (typically to a dose of 1 kGy) in the presence of oxygen. Total hydroperoxide concentrations in these solutions were measured using the xylenol orange assay described by Wolff,[16] after removal of hydrogen peroxide by addition of catalase; the results are summarised in Table 1. A mixture of hydroperoxides is evidently formed from thymine (predominately the 5- and 6-TOOH); the remaining pyrimidine compounds have been shown to generate analogous hydroperoxides,[3,10] but at considerably lower yields. No attempts were made to separate or identify the individual hydroperoxides.

It was not possible to determine the amount of hydroperoxides generated from DNA and RNA due to difficulties arising from their side reactions with the xylenol orange complex as reported by Michaels and Hunt.[17]

(b) Reaction of pyrimidine hydroperoxides with Fe^{2+}

EPR spectra were recorded of reaction mixtures of hydroperoxides with Fe^{2+}–EDTA in the presence of the spin-traps MNP or DMPO, throughout the pH range 1–7.4. Concentrations (after mixing) were typically; Fe^{2+}–EDTA 1 : 1 complex (1×10^{-3} mol dm^{-3}), hydroperoxide (in the range of 2×10^{-5} to 1×10^{-4} mol dm^{-3}) and either MNP (5×10^{-3} mol dm^{-3}) or DMPO (0.05 mol dm^{-3}).

(i) Nucleobases. The reaction of uracil hydroperoxide with Fe^{2+}–EDTA in the presence of DMPO, resulted in the detection of signals from two spin adducts, throughout the pH range employed (see Fig. 1). These signals are assigned to adducts formed by trapping a carbon-centred radical [a(H) = 1.60. a(H) = 2.32 mT] and an oxygen-centred radical. [a(N) = 1.58, a(H) = 1.72 mT], on the basis of their hyperfine coupling constants. Solvent extraction experiments based on methods described by Kalyanaraman *et al.*,[18] provided further evidence for the assignment of the oxygen-centred species to an alkoxyl radical: extraction of the spin-adduct into toluene showed that the EPR parameters of the signal assigned to the carbon-

Table 1 The concentrations of hydroperoxides detected from the pyrimidine and purine nucleobases and nucleosides following irradiation

Substrate	Conc./μmol dm^{-3} [a]
Uracil	33.2
Uridine	49.4
Cytosine	36.7
Cytidine	40.6
Thymine	148.2
Thymidine	88.4
Adenine	12.7
Adenosine	15.3
Guanine	8.9
Guanosine	13.2

[a] Concentration of peroxide ±5.0%. For conditions see text.

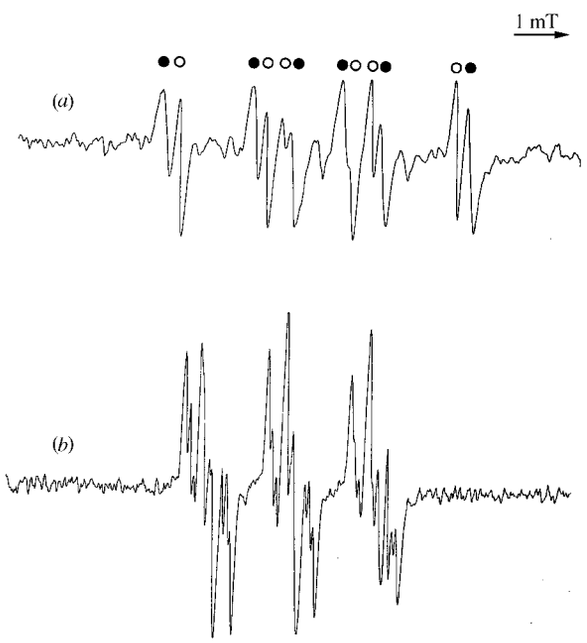


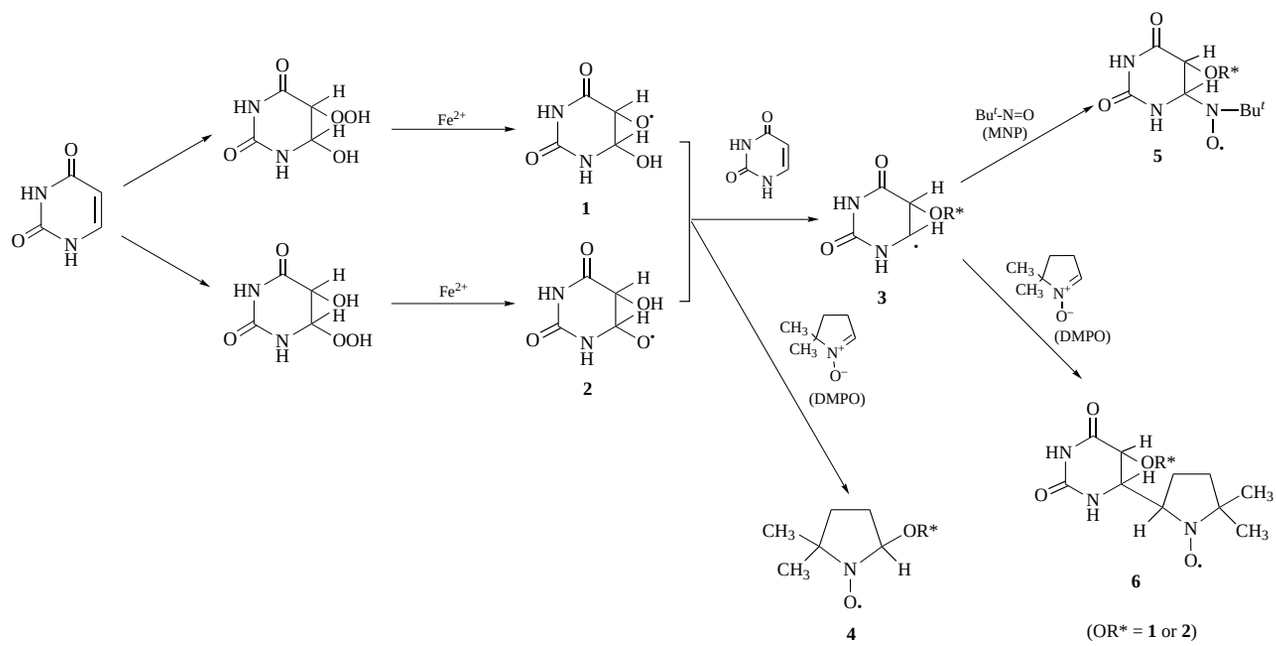
Fig. 1 (*a*) EPR spectra of spin adducts **6** (●) and **4** (○) formed from the degradation of uracil hydroperoxide (2.21×10^{-5} mol dm^{-3}) on reaction with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) in the presence of DMPO (0.05 mol dm^{-3}) (for parameters see Table 2); (*b*) as (*a*) except with MNP (5×10^{-3}), signals are assigned to spin adduct **5**

centred species did not change dramatically, whereas the signal assigned to the oxygen-centred radical altered considerably [to a(N) = a(H) = 1.47 mT]. These differences are attributed to conformational changes of the DMPO–OR spin-adduct, due to a loss of hydrogen-bonding interaction on going from an aqueous environment to toluene.[18]

Parallel experiments with MNP gave a strong and characteristic EPR spectrum (Fig. 1), showing the presence of a single adduct, with a large triplet [a(N) = 1.50 mT] and further partially resolved nitrogen [a(β–N) = 0.35 mT] and hydrogen [a(β–H) = 0.14 and a(γ–H) = 0.07 mT] splittings (see Table 2). Such a spectrum is characteristic of the trapping of a C(6)-yl adduct of uracil, and is similar, but not identical, to spectra observed following hydroxyl or alkoxyl radical (Bu^{t}O$^{\bullet}$) attack on uracil itself.[19,20]

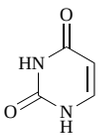
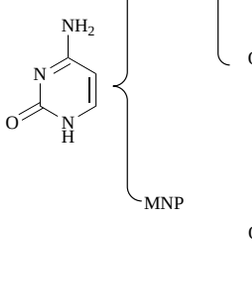
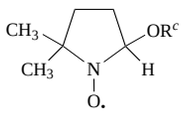
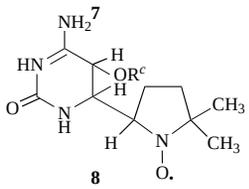
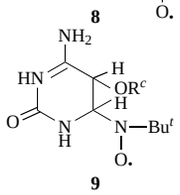
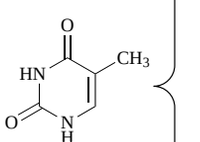
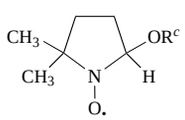
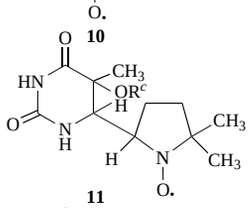
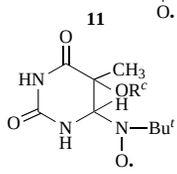
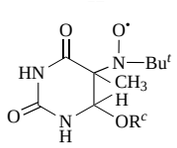
We assign the MNP-adduct to radical (**5**) (see Scheme 1), formed from addition of alkoxyl radical (**1**) and/or (**2**) to an excess of unreacted uracil (not converted to hydroperoxide, during irradiation) to give (**3**) which is subsequently trapped by MNP [signals from the isomeric C(6)-adducts identified with uridine (see later), may be present in relatively low concentrations]. Experiments with excess of added pyrimidines showed that these substrates were attacked in competition with the parent substrates (see later). With DMPO, direct trapping of the alkoxyl radicals themselves [(**1**) or (**2**)] gives rise to (**4**) (see Scheme 1), whereas the carbon-centred species is assigned to (**6**), arising from the subsequent trapping by DMPO of species (**3**), which again is formed by addition of (**1**) or (**2**) to the parent nucleobase. The possibility that (**6**) may be a radical derived from intramolecular rearrangement of the alkoxyl radicals [(**1**) or (**2**)] cannot be ruled out entirely; however, experiments in which additional amounts of the parent compounds were added, resulted in spectra which showed an increase in the intensity of the carbon-centred adduct signals, at the expense of the oxygen-centred species, consistent with the formation of (**6**) by trapping of (**3**). If these assignments are correct, then the initial radicals [(**1**) and (**2**)] themselves do not undergo rapid rearrangement (*e.g.* 1,2-shifts) or fragmentation, in contrast to the behaviour of other alkoxy radicals from alkyl hydroperoxides.[21]

With cytosine hydroperoxide, the spectra obtained with DMPO also indicated the formation of oxygen- and carbon-centred species (**7**) and (**8**) (see Table 2); with MNP, the spectrum (see Fig. 2) reveals the presence of a C(6)-yl species (**9**),



Scheme 1

Table 2 EPR parameters of radicals derived from the metal-catalysed degradation of nucleobase hydroperoxides in the presence of the spin traps MNP and DMPO, in the pH range 1–7.4

γ-Irradiated substrate[b]	Spin-trap	Radicals	*a*(N)[a]/mT	*a*(other)[a]/mT
	DMPO	**4**	1.58	1.72 (β-H)
		6	1.60	2.32 (β-H)
	MNP	**5**	1.50	0.35 (β-N) 0.14 (β-H) 0.07 (γ-H)
	DMPO	 **7**	1.56	1.72 (β-H)
		 **8**	1.61	2.32 (β-H)
	MNP	 **9**	1.50	0.33 (β-N) 0.14 (β-H)
	DMPO	 **10**	1.56	1.89 (β-H)
		 **11**	1.61	2.32 (β-H)
	MNP	 **12**	1.55	0.36 (β-N)
		 **13**	1.60	

[a] Typically ±0.005 mT, except where indicated otherwise; $g = 2.0059 \pm 0.0001$. [b] See text for further details. [c] OR = Alkoxyl radicals from the C(5) or C(6) hydroperoxide adduct of pyrimidine.

presumably an alkoxyl radical adduct, as seen with the uracil hydroperoxides.

Analogous results were observed with thymine hydroperoxide(s) in the presence of DMPO [see Table 2 with reference to (**10**) and (**11**)]; experiments with MNP gave a spectrum (see Fig. 3) in which the signal from the C(5)–OR adduct (**12**) is dominant [with some evidence of a small contribution from the C(6)–OR adduct (**13**); *cf.* reactions with $HO^{\bullet}$].[20]

The degradation experiments were next conducted in the presence of high concentrations of an additional nucleobase or nucleoside. For example, when thymine hydroperoxide (*ca.* 1.48×10^{-3} mol dm^{-3}), was degraded by Fe^{2+} in the presence of large amounts of uracil (5×10^{-2} mol dm^{-3}), the spectrum obtained (using MNP) showed the presence of signals with splittings identical to (**5**) (described above), which are clearly assigned to the C(6)-yl species of uracil (**16**), formed from addition of thymine alkoxyl radicals [(**14**) or (**15**)] to excess uracil, instead of thymine itself.

(ii) Nucleosides. The reaction of uridine hydroperoxide with Fe^{2+} in the presence of MNP resulted in the detection of a very characteristic spectrum at pH 7.4, as shown in Fig. 4. The signals are analysed in terms of a triplet of doublets split further into a triplet [*a*(N) = 1.5, *a*(H) = 0.16 and *a*(N) = 0.26 mT] and a triplet of doublets [*a*(N) = 1.51 and *a*(H) = 0.35 mT], *cf.*

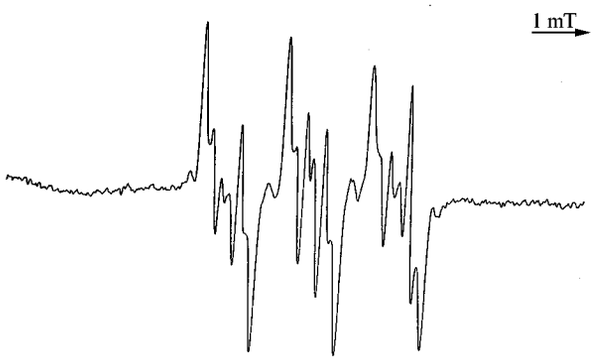


Fig. 2 EPR spectrum of the spin adduct **9** formed from the degradation of cytosine hydroperoxide (2.45×10^{-5} mol dm^{-3}) on reaction with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) in the presence of MNP (5×10^{-3}) (for parameters see Table 2)

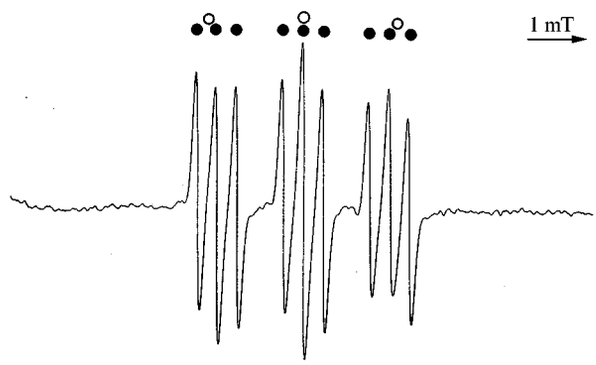
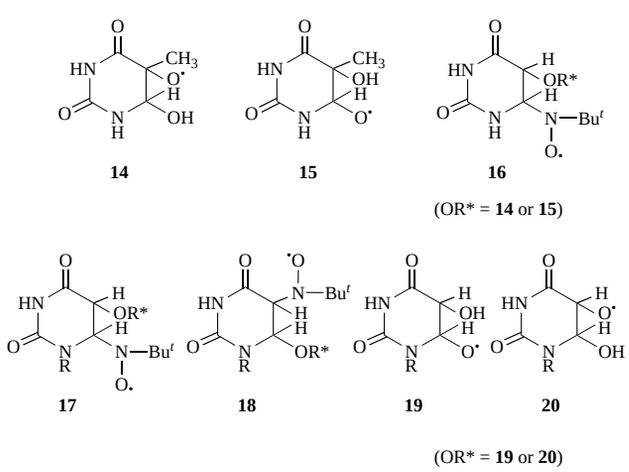


Fig. 3 EPR spectra of spin adducts **12** (●) and **13** (○) formed from the degradation of thymine hydroperoxide on reaction with Fe^{2+}–EDTA (1×10^{-3}) and thymine hydroperoxide (9.88×10^{-5} mol dm^{-3}) in the presence of MNP (5×10^{-3} mol dm^{-3}). A minor contribution from spin adduct **13** (○) is believed to be present (for parameters see Table 2).



HO$^{\bullet}$ adducts with uridine.[22] These are attributed to the spin-trapped C(5)–OR (**17**) and C(6)–OR (**18**) adducts, arising from the addition of alkoxyl radical (**19**) and/or (**20**).

With DMPO, signals were detected whose parameters (Table 3) are analogous to those obtained from the nucleobase hydroperoxides, indicating the presence of a carbon-centred adduct and an alkoxyl radical.

This pattern of alkoxyl radical attack across the C(5)–C(6) double bond, to give the respective alkoxyl-adducts, was also observed following the degradation of cytidine and thymidine hydroperoxides (see Table 3 for parameters); studies with DMPO gave similar spectra to those observed with the nucleobase hydroperoxides. Their reactions in the presence of MNP

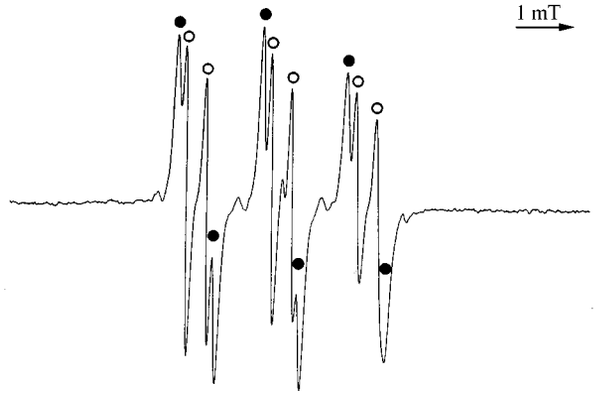


Fig. 4 EPR spectra of spin adducts **17** (●) and **18** (○) formed from the degradation of uridine hydroperoxide (3.29×10^{-5} mol dm^{-3}) on reaction with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) in the presence of MNP (5×10^{-3} mol dm^{-3}). Only the outside lines of **17** are indicated (see Table 3 for further details).

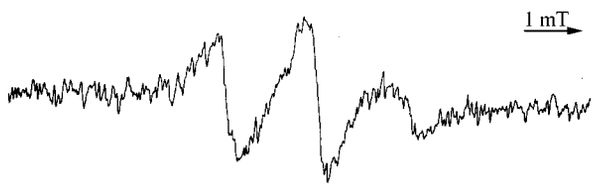


Fig. 5 EPR spectrum of spin adduct formed from the degradation of DNA hydroperoxide [concentrations unknown (see text)] on reaction with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) in the presence of MNP (5×10^{-3} mol dm^{-3})

resulted in spectra which again gave signals showing the presence of C(5)- and C(6)-yl radicals, arising from alkoxyl radical addition.

(c) Reaction of purine hydroperoxides with Fe^{2+}

When purine nucleobases and nucleosides were exposed to high energy electrons in the presence of oxygen, hydroperoxides were also generated; however, their exact structures and relative concentrations were not determined. When the hydroperoxide mixtures were decomposed by reaction with Fe^{2+}, the spin-adducts were only detected in the presence of DMPO, of which the signals are attributed to carbon-centred adducts [a(N) = 1.60 and a(H) = 2.32 mT]. Attempts to study this further by employing MNP resulted in the detection of di-*tert*-butyl aminoxyl, arising from the decomposition of the trap. The low solubility of these materials has contributed to difficulties in producing detectable levels of base-derived adducts from other spin-traps. However, when these reactions were conducted in the presence of pyrimidines (2×10^{-2} mol dm^{-2} mol dm^{-3}), this resulted in the detection of alkoxyl adducts on the base (with MNP). These results indicate that alkoxyl radicals are indeed formed following the degradation of the purine hydroperoxides and that these radicals undergo addition reactions similar to those observed with the pyrimidine hydroperoxides.

(d) Reaction of DNA and RNA hydroperoxides with Fe^{2+}

DNA and RNA (2 mg cm^{-3}) samples which had been exposed to relatively low doses of radiation (330 and 660 Gy), were treated with Fe^{2+} at pH 7.4 in the presence of MNP. The spectra obtained (see Fig. 5) gave broadened isotropic signals [a(N) = 1.50 mT] in each case. When DNA and RNA samples were exposed to larger irradiation doses (1 kGy), in the expectation that more hydroperoxides would be generated, similar results were obtained.

Reactions in the presence of DMPO gave spectra which showed the presence of only adducts of carbon-centred radicals [a(N) = 1.61 and a(H) = 2.32 mT]. Although it is not possible to identify the structure of these adducts, it is believed that they

Table 3 EPR parameters of radicals derived from the metal-catalysed degradation of pyrimidine nucleoside, purine nucleobase and nucleoside, RNA and DNA hydroperoxides in the presence of DMPO and MNP

μ-Irradiated Substrate[b]	Spin-Trap	Radicals		a(N)[a]/mT	a(other)[a]/mT
Uridine	MNP	C(5)–OR–C(6)–yl–radical	(**17**)	1.50	0.16 (β-H) 0.26 (β-N)
		C(6)–OR–C(5)–yl–radical	(**18**)	1.51	0.35 (β-H)
	DMPO	Alkoxyl adduct		1.58	1.72 (β-H)
		C(5)–OR–C(6)–yl–radical or C(6)–OR–C(5)–yl–radical		1.60	2.32 (β-H)
Cytidine	DMPO	Alkoxyl adduct		1.56	1.72 (β-H)
		C(5)–OR–C(6)–yl–radical or C(6)–OR–C(5)–yl–radical		1.61	2.32 (β-H)
	MNP	C(5)–OR–C(6)–yl–radical		1.48	0.16 (β-H)
		C(6)–OR–C(5)–yl–radical		1.48	0.26 (β-N)
Thymidine	DMPO	Alkoxyl adduct		1.56	1.89 (β-H)
		C(5)–OR–C(6)–yl–radical or C(6)–OR–C(5)–yl–radical		1.61	2.32 (β-H)
	MNP	C(5)–OR–C(6)–yl–radical		1.43	0.30 (β-H)
		C(6)–OR–C(5)–yl–radical		1.60	
Guanine	DMPO	Carbon-centred radical		1.60	2.32 (β-H)
Adenine	DMPO	Carbon-centred radical		1.60	2.32 (β-H)
Guanosine	DMPO	Carbon-centred radical		1.60	2.32 (β-H)
Adenosine	DMPO	Carbon-centred radical		1.60	2.32 (β-H)
RNA	DMPO	Carbon-centred radical		1.61	2.32 (β-H)
	MNP			1.50	
DNA	DMPO	Carbon-centred radical		1.61	2.32 (β-H)
	MNP			1.50	

[a] Typically ±0.005 mT, except where indicated otherwise; $g = 2.0059 \pm 0.0001$. [b] See text for further details.

are derived from alkoxyl radical attack on the bases from DNA and RNA itself (either on the same strand or the alternate strand in the case of DNA). The latter type of reaction might be expected to give rise to interstrand cross-links which may be of biological significance as they may be difficult to repair. The lack of alkoxyl radical DMPO adducts may reflect the rapidity of this process.

(e) Reaction of hydroperoxides with Fe^{2+} in the presence histone proteins and selected model compounds

These studies were extended to investigate the possible transfer of damage from alkoxyl radical generation to histone proteins. Such a process could be of major biological importance, as these proteins, usually associated with DNA are believed to be responsible for maintenance of chromatin and nucleosome structure, as well as regulating the availability of DNA for replication and transcription.[23] Histone proteins are rich in basic amino acids, with the positive charges on these residues interacting with the negatively charged phosphate groups on the DNA double helix.

Pyrimidine, purine, RNA and DNA hydroperoxides were treated with Fe^{2+}, as before, in the presence of both a histone protein (typically, histone IIA, IIS and 3, in the range of 1–3×10^{-3}) and MNP.

Spectra with somewhat broadened isotropic signals were obtained [a(N) = 1.51 mT] which indicate the presence of histone-derived radicals, with all the hydroperoxides except cytosine and cytidine, for which no signals were observed. Fig. 6(*a*) shows the spectrum obtained following the degradation of thymine hydroperoxide in the presence of histone IIS, with MNP, showing isotropic signals [a(N) = 1.51 mT] labelled (●) and anisotropic features (○). Similar studies with histone IIA [Fig. 6(*b*)] gave spectra showing broadened isotropic signals [a(N) = 1.50 mT] but further splittings could not be resolved. For each individual histone studied, the signals obtained appeared to be similar, irrespective of the type of hydroperoxide employed; for example, it was not possible to differentiate between attack from thymine or uracil hydroperoxide.

These findings suggest that alkoxyl radicals, generated from the hydroperoxides are capable of reacting with the histone proteins (presumably *via* hydrogen abstraction). A possible explanation for the absence of any reaction with cytosine and cytidine hydroperoxides may be repulsion between the positive charges of these hydroperoxides and those of the histone proteins.

Similar investigations with added amino acids (typically in the range of 1–3×10^{-3} mol dm^{-3}) and in the presence of MNP, were also shown to generate radicals *via* hydrogen abstraction by hydroperoxide-derived alkoxyl radicals. For example, spectra obtained by the reaction of thymine hydroperoxide and Fe^{2+} in the presence of L-serine and the tripeptide Gly-Gly-Gly respectively, are shown in Fig. 7. For the former, the signals [a(N) = 1.50 and a(H) = 0.18 mT] are attributed to the adduct of [H_3N^+–CH($\dot{C}$HOH)–COO^-], evidently formed by hydrogen atom abstraction by the alkoxyl radical. Reactions of Gly-Gly-Gly gave a trapped radical with β-H and β-N splittings [a(N) = 1.57, a(H) = 0.24 and a(β-N) = 0.24 mT], which indicate that the species has the structure [–NH–$\dot{C}$H–CO–]; such radicals are believed to be formed by hydrogen atom abstraction from either the middle or C-terminal α-carbons of Gly-Gly-Gly.[24] This reaction is of great significance, as these α-carbon radicals are essential precursors to the breakage of the peptide backbone; therefore, formation of these species on the histones may lead to protein fragmentation. Similar spectra were observed with other hydroperoxides, and analogous reactions were observed with other amino acids such as D,L-leucine and

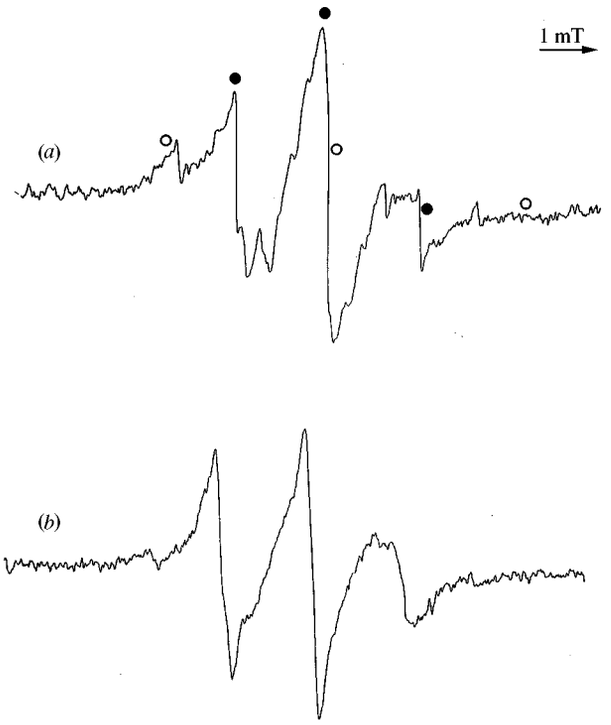


Fig. 6 (*a*) EPR spectra of the spin adducts formed from the transfer of damage to histone IIS (1×10^{-3} mol dm^{-3}), following the degradation of thymine hydroperoxide (9.88×10^{-5} mol dm^{-3}) on reaction with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) in the presence of MNP (5×10^{-3} mol dm^{-3}): isotropic signals (●); anisotropic signals (○). Signals are believed to arise from alkoxyl radical attack (derived from the thymine hydroperoxide) on histone IIS (see text for further details); (*b*) as in (*a*) except with histone IIA.

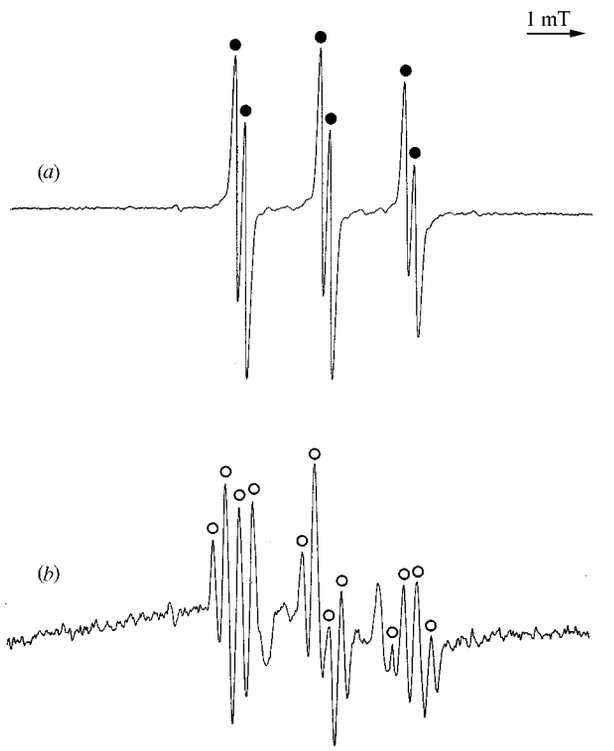


Fig. 7 (*a*) EPR spectra of the spin adducts formed from the transfer of damage to L-serine (1×10^{-3} mol dm^{-3}), following the degradation of thymine hydroperoxide (9.88×10^{-5}) on reaction with Fe^{2+}–EDTA (1×10^{-3}) in the presence of MNP (5×10^{-3} mol dm^{-3}). Signals marked (●) are assigned to the trapped radical [H_3N^+–CH(ĊHOH)–COO^-]; (*b*) as in (*a*) except with Gly-Gly-Gly; signals marked (○) are assigned to an adduct with the structure [–NH–ĊH–CO–] (see text for further details).

L-proline. For example with D,L-leucine, the dominant signal comprises a triplet of doublets [a(N) = 1.50 and a(H) = 0.18 mT], indicative of the trapping of a secondary carbon-centred radical and is attributed to side-chain attack to give {H_3N^+–CH-[ĊHCH(CH_3)$_2$]–COO^-}. With L-proline, the signals are dominated by a triplet [a(N) = 1.51 mT] of doublets [a(H) = 0.28 mT] of doublets [a(H) = 0.10 mT], evidently from attack at one or more of the methylene groups on the side-chain. These examples show that hydrogen abstraction occurs at both the back-bone and side-chains, as is also observed in EPR spin-trapping studies with HO$^\bullet$.[24]

(f) Hydroperoxide reactions with Fe^{2+} in the presence of antioxidants

Reactions of the hydroperoxides with Fe^{2+} were carried out in the presence of antioxidants which included reduced glutathione (GSH), cysteine and ascorbic acid; experiments were conducted in the absence and presence of spin-traps (MNP and DMPO), under the same conditions as before.

In the presence of GSH (5×10^{-3} mol dm^{-3}), the degradation of all the hydroperoxides by Fe^{2+} gave a spectrum (with DMPO) with signals [a(N) = 1.55 and a(H) = 1.60 mT] indicating the formation of a thiyl-radical from GSH[24] [see reactions (1) and (2)]. Studies with cysteine resulted in the detection of

$$RO^\bullet + GSH \longrightarrow ROH + GS^\bullet \quad (1)$$

$$GS^\bullet + DMPO \longrightarrow GS\text{–}DMPO^\bullet \quad (2)$$

similar thiyl-derived signals [a(N) = 1.50 and a(H) = 1.70 mT].[25] Reactions with the ascorbate anion (1×10^{-2} mol dm^{-3}) in the absence of any spin-trap, resulted in the detection of a characteristic doublet signal [a(H) = 0.176 mT] assigned to the ascorbyl radical, arising from oxidation of the antioxidant by hydroperoxide-derived radicals.

Experiments with all three antioxidants (1×10^{-2} mol dm^{-3}) in the presence of the pyrimidine hydroperoxides and MNP resulted in the detection of reduced concentrations (*ca.* 50%) of the characteristic alkoxyl radical-adducts seen with the hydroperoxides themselves following reaction with Fe^{2+} (see earlier). When antioxidant concentrations were increased (in the range 1×10^{-2} to 0.1 mol dm^{-3}), the alkoxyl-adduct signals decreased further in intensity. These results provide further evidence that the alkoxyl radicals can undergo hydrogen abstraction and that these reactions occur readily with compounds which are readily oxidised, as indicated by the antioxidants employed in these studies.

If we assume that the first-formed pyrimidine alkoxyl radicals react with DMPO at the same rate as that reported for Bu^{t}O$^\bullet$ (9×10^6 dm^3 mol^{-1} s^{-1}),[26] then we can estimate the rate constant for attack of the alkoxyl radical on the pyrimidines themselves. For example, in the case of uracil the observation of both alkoxyl adducts and carbon-centred radical-adducts (from attack of RO$^\bullet$ on uracil) for concentrations of DMPO and uracil of 0.05 and 1×10^{-2} mol dm^{-3} respectively, suggests that the rate constant for attack on the latter is *ca.* 5×10^7 dm^3 mol^{-1} s^{-1} (see ref. 27 for comparable results for the reaction between Bu^{t}O$^\bullet$ and thymidine). Given the observation that the reaction of RO$^\bullet$ (R = pyrimidine) with GSH gives GS$^\bullet$, but not trapped RO$^\bullet$ or R$^\bullet$, we estimate the rate constant of the reaction between RO$^\bullet$ and GSH as *ca.* 1×10^8 dm^3 mol^{-1} s^{-1} (substantially faster than that of Bu^{t}O$^\bullet$).[27] No evidence for rearrangement reactions (*e.g.* 1,2-shifts) or fragmentation (*e.g.* β-scission reactions) of RO$^\bullet$ (unlike Bu^{t}O$^\bullet$) has been obtained for which an upper limit for k can be estimated as *ca.* 10^6 dm^3 mol^{-1} s^{-1}.

Conclusions

EPR spin-trapping results are consistent with the formation of alkoxyl radicals on degradation of these hydroperoxides by

Fe^{2+}–EDTA. The alkoxyl radicals have been shown to react with the nucleobases and nucleosides themselves, as well as with antioxidants, histone proteins and amino acids. The spectra obtained following alkoxyl radical attack on parent and added pyrimidine compounds are comparable with the reactions of other alkoxyl (particularly $Bu^tO^{\bullet}$)[19] radicals, for which addition to the C(5)–C(6) double bond of the base is observed. The alkoxyl radicals show a preference to react with other substrates, as opposed to undergoing intramolecular rearrangement and fragmentation, as is observed with many other alkyl hydroperoxides.[21] Such intermolecular reactions may account for the known mutagenicity of these hydroperoxides particularly in the presence of metal ions such as Fe^{2+} and Cu^{2+}.

Evidence has also been obtained for reaction of the alkoxyl radicals with histone proteins and antioxidants. The former process would be expected to result in an increased extent of biological damage; the latter finding suggests that cellular antioxidants would be expected to ameliorate the damage induced by these species. Experiments with the purine, DNA and RNA hydroperoxides confirm alkoxyl radical formation and reaction, providing evidence for damage to these systems; the generation and reactions of alkoxyl radicals from RNA and DNA hydroperoxides may play a role in the formation of DNA double-strand breaks and inter-strand crosslinks.[14,15]

Experimental

Stock solutions of the DNA nucleobases and nucleosides were prepared in (1×10^{-2} mol dm^{-3}) phosphate buffer pH 7.4 for radiolysis. Typical substrate concentrations used were 1×10^{-2} mol dm^{-3} and the solutions were irradiated in the presence of oxygen to a total dose of 1 kGy, at a dose rate of 66 Gy min^{-1} using a 2.5 MeV van de Graff electron accelerator fitted with a 5% beam attenuator operating in a vertical mode. All solutions were exposed for 15 min in total; during this time, irradiations were halted every few minutes to allow the samples to be reoxygenated. After irradiation, 5 μl of catalase (2 mg cm^{-3}) was added per cm^3 of irradiated solution to degrade the H_2O_2 generated during irradiation. Solutions were left at room temperature for a further 10 min and then frozen in liquid nitrogen.

UV–VIS experiments were conducted on a Hitachi U3000 spectrophotometer and hydroperoxide concentrations were measured using the xylenol orange assay described by Wolff.[14] EPR experiments were carried out on either Bruker ESP300 or JEOL JES RE1X EPR spectrometers, using an aqueous solution sample cell. In these experiments where the hydroperoxides were degraded, samples were prepared by mixing the hydroperoxide solutions (usually in the range 2×10^{-5} to 1×10^{-4} mol dm^{-3}) with Fe^{2+}–EDTA (1×10^{-3} mol dm^{-3}) which were deoxygenated by purging with nitrogen, in the presence of either DMPO (0.5 mol dm^{-3}) or MNP (5×10^{-3}); all solutions were prepared in deionised water, and the MNP contained (<15% v/v) acetonitrile to bring about dissolution to the trap. Variation of pH was achieved by addition of H_2SO_4 or NH_3

Irradiation experiments were carried out at the Cookridge Radiation Centre (Leeds), and all chemicals were obtained from Sigma and/or Aldrich. Chemicals were used without further purification, except DMPO, which was purified by treatment with activated charcoal.

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